IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re

Patent Application of

Byron E. Anderson

Applicant: Bio Science International, Inc.

Patent Application No. 10/612,298

Filed: July 2, 2003

Examiner: Mark Shibuya

For: "Peptides comprising aromatic D-amino acids
and methods of use"

DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

The undersigned hereby declares and states:

1. I am the inventor of the subject matter of all the currently pending claims (*i.e.*, claims 1-45) of the above-identified patent application. I have assigned all rights, title and interest in the above-identified application to Bio Science International, Inc. This Declaration is submitted in furtherance of prosecution of the above-identified application.

2. My graduate training was in biological chemistry, more specifically in protein structure-function, at the University of Michigan and Johns Hopkins University. My postdoctoral studies were in the field of immunochemistry, more specifically the nature of binding of ligands to antibodies, at Columbia University, the College of Physicians and Surgeons. I had a position for 32 years at Northwestern University Medical School, Chicago, IL, with appointments in

several departments, primarily the Biochemistry Department, and I retired in 2002 as a Professor Emeritus. I am also a co-founder of three biotech companies, including: Immtech Pharmaceuticals, Septech, Inc., and Bio Science International, Inc., the assignee of the above-identified patent application.

3. My knowledge of the subjects of peptide and protein structure and function and carbohydrate structure and function are, in part, derived from teaching these subjects for 30 years to graduate and medical students. Also, I have worked extensively with, conducted research on, and written papers related to the biological activities of various peptides, proteins and complex carbohydrates, more specifically, on the modulation of biological activities of proteins through interaction with other molecules. I consider myself an expert in the field of protein chemistry and the study of peptides. A copy of my Curriculum Vitae is attached herein as Exhibit A.

4. I have reviewed the Office Action, dated July 3, 2006, concerning the above-identified patent application. I understand that while claims 1-45 of the above-identified application are pending, claims 5-9 and 43-45 are under consideration and claims 1-4 and 10-42 have been withdrawn from consideration. I further understand that this declaration is being filed to overcome anticipation rejections in view of Dooley et al. (Science 266:1019-2022 (1994)) and Pinilla et al. (J. Mol. Biol. 293:1013-1025 (1998)), and obviousness rejections in view of various combinations of Dooley et al., Pinilla et al., Lam et al. (Nature 354:82-84 (1991)), Lebl et al. (Biopolymers 37:177-198 (1995)) and Satoh et al. (Analytical Biochemistry 260:96-102 (1998)).

5. More specifically, I understand that this declaration is being filed to respond to the Examiner's assertion that Dooley et al. "teach, throughout the document and especially the abstract and table 1, column c, the preparation of libraries of D-amino acid containing heptapeptides (7 residues)," including an "Ac-rtfwyxx-NH2 library, which contains fixed

residues including D-tryptophan (w), D-tyrosine (y), and D-phenylalanine (f) in 100% of the species in the library," and thus, "reads on all the limitations of claims 5 and 6." I also understand that the Examiner has asserted that Pinilla et al. "teach, throughout the document and especially table 1, an all-D amino acid containing hexapeptide library," including an "Ac-yryxxx-NH2 library, which contains two fixed D-tyrosine (y) residues plus three random positions (including D-tyrosine), such that 74% (20 of 27) of the species in the library have three D-tyrosine residues," and thus, "reads on the limitations set forth in claims 5 and 6."

6. I have reviewed the claims of the above-identified application and understand claims 5-9 and 43-45 are directed generally to a "library comprising a plurality of D-peptides, wherein each D-peptide comprises from three to seven D-amino acid residues" and wherein at least 25% of the D-peptides "comprise at least three amino acid residues independently selected from the group consisting of D-tryptophan, D-tyrosine, and D-phenylalanine." I have also reviewed the Dooley et al., Pinilla et al., Lam et al., Lebl et al. and Satoh et al. references, as well as other published research papers from these investigators.

7. With respect to the Examiner's assertion that Dooley et al. disclose libraries of D-amino acid containing heptapeptides, including an Ac-rtfwyxx-NH2 library containing fixed residues of D-tryptophan (w), D-tyrosine (y), and D-phenylalanine (f) in 100% of the species in the library, I would like to clarify that the description of Table 1 on page 2020 provides that Dooley et al. were, more precisely, measuring "[i]nhibition of [^{3}H]DAMGO binding by peptide mixtures derived from the acetylated SPCL," (emphasis added), and the columns of Table 1 provide the IC_{50} values of the inhibitory peptide mixtures obtained at each step of the "iterative selection process," which was carried out on the most active mixtures in order to define each of the four mixture positions. (*See* Dooley et al., fn 8 and 9). At each iteration, the number within

each mixture was reduced by a factor of 19." (*See id.* at Table 1 and fn 8 and 9).

8. I respectfully submit that Dooley et al. does not disclose a library comprising a plurality of D-peptides, wherein each D-peptide comprises from three to seven D-amino acid residues and wherein at least 25% of the D-peptides comprise at least three amino acid residues independently selected from the group consisting of D-tryptophan, D-tyrosine, and D-phenylalanine because Table 1 discloses peptide mixtures, not peptide libraries as asserted by the Examiner. The reasoning for my assertion in the preceding sentence is as follows, and is based on (a) the interpretations of what constitutes a library by the Dooley et al. and Pinilla et al. authors, and (b) the differences between the aromatic residue contents of the Dooley and Pinilla et al. libraries as compared to the libraries of the present patent application.

a. The authors of the Dooley et al. and Pinilla et al. references, as well as other papers written by these investigators, all clearly define what constitutes their "libraries." For example, Clemencia Pinilla and C. T. Dooley have described its synthetic combinatorial library (SCL) or synthetic peptide combinatorial library (SPCL) as 34 million peptides (*see* Houghten, R.A., et al., Nature 354:84-86 (1991) (of which both Dooley and Pinilla are authors); Pinilla et al.) or the 52,128,400 peptides of the other articles, and that they are composed of 400 mixtures from which an iterative (*i.e.*, repetitive) selection process is used to define the most active peptides within the mixtures of the SPCL. (*See* Dooley et al., Fig. 1). In fact, Pinilla et al. stated that they used "an SPCL composed in total of 52 128 400 nonacetylated hexapeptides," "[t]herefore, each of the 400 different mixtures that makes up this library," (Pinilla et al., Gene 128:71-76 (1993)), and the "SCL was composed of 400 different hexapeptide mixtures" (Pinilla et al., Peptide Research 8:250-257 (1995)). Likewise, Dooley et al. have referred to its "400 peptide mixtures making

up this SCL," and has stated that the "peptide library used in this study is made up of 400 mixtures" each of which is composed of "130,321 hexapeptides (19 to the 4th power)," and in "total the library contains 52,128,400 hexapeptides (400 x 130,321)." (Dooley et al., Proc. Nat. Acad. Sci. 90:10,811-10,815 (1993)). Further, the Dooley et al. and Pinilla et al. references cited by the Examiner provide that the definition of the library refers to the 52 or 34 million member peptide libraries (*see* Dooley et al., Fig. 1 ("The library consists of 400 mixtures; each mixture contains 130,321 peptides; the SCL in total is made up of 52,128,400 different peptides"); Pinilla et al., p. 1021 ("The hexapeptide PS-SCL consists of 120 peptide mixtures having one position individually defined with 20 D-amino acids (represented by o) and the remaining five positions as mixtures (represented as x)"), the latter being the same number of peptides as used in the Houghten et al. reference discussed above.

In the Pinilla et al. paper, the repetitive, iterative process of selection is described (*see* Pinilla et al., p. 1014 ("At position 1 of the library, only mixtures defined with phenylalanine . . . were found to have significant activity. The second and third positions were clearly specific for mixtures . . . . The mixture with tyrosine defined at position 4 was the most active . . . . Positions 5 and 6 were less specific in that mixtures . . . ."). Therefore, for each of the iterative steps, the peptide populations are described as mixtures. (*See* Dooley et al., fn 8 ("For each iteration, 20 new mixtures are synthesized such that one of the x positions is individually defined")). The iterative method is a repetitive selection process whereby subpopulations of the library are identified by whatever biologic assay is pertinent to the inquiry of the study. The subpopulations, or the mixtures, are not libraries themselves. In all of the papers I have cited, or in Dooley

et al. and Pinilla et al., the authors could have selected in the iterative process any of all the possible combinations of amino acid sequences.

b. With regard to the aromatic amino acid contents of the libraries of the above-identified patent application compared to that of Dooley et al. and Pinilla et al., the percentage of hexapeptide sequences containing 3 or more of the aromatic amino acids (phenylalanine, tyrosine and/or tryptophan), in the libraries of the Dooley et al. and Pinilla et al. references, and the percentage of such sequences of tri- to hepta-peptides as specified in claim 5 of the patent application, are readily calculated: The percentage of sequences in both the Dooley and Pinilla libraries that would contain 3, 4, 5 or 6 phenylalanine, tyrosine, and/or tryptophan residues, is calculated as 4.88%. For example, in the Dooley et al. library, 2,543,049 peptide sequences (of the 52,148,400 total sequences) contain 3 or more of the aromatic amino acids phenylalanine (phe), tyrosine (tyr) and/or tryptophan (trp).

Therefore, the libraries as specified in claim 5 are unique in their properties of containing high percentages of the aromatic residues specified; the unique properties of the individual aromatic residues account, in theory, and in practice as shown by the examples of the patent application, in yielding certain members of such libraries with high binding affinities and specificities to the proteins tested.

9. In response to the Examiner's assertion that Pinilla et al. teach an all-D amino acid containing hexapeptide library, including an Ac-yryxxx-NH2 library, which contains two fixed D-tyrosine residues plus three random positions, such that 74% of the species in the library have three D-tyrosine residues, I would like to point out to the Examiner that Table 1 does not disclose a peptide library. Pinilla et al. have identified 27 peptide sequences individually. (*See* Pinilla et

al., "Table 1. Most active amino acids selected from PS-SCL", where PS-SCL is equal to positional scanning of the synthetic combinatorial library that were synthesized individually, and then tested in three different systems (*i.e.*, for inhibitory activity of three different monoclonal antibodies)). These are individual peptides that are grouped into this one table and they clearly do not constitute a library. (*See* Pinilla et al., p. 1015 ("The combination of these amino acids resulted in the synthesis of 27 individual all-D hexapeptides.")). Further supporting the assertion that the 27 peptides are not a library is that the 27 peptides are not together in the same vessel and then probed by some method to identify a positive reaction, as is the use of libraries of any type of chemical compound. In the above-noted patent application, the members of the specified libraries are probed by described assay systems.

10. Thus, I submit that because Dooley et al. and Pinilla et al. do not disclose a library comprising a plurality of D-peptides, wherein each D-peptide comprises from three to seven D-amino acid residues and wherein at least 25% of the D-peptides comprise at least three amino acid residues independently selected from the group consisting of D-tryptophan, D-tyrosine, and D-phenylalanine, and, therefore, Dooley et al. and Pinilla et al. do not anticipate claim 5.

11. I hereby declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: January 21, 2007

Byron E. Anderson

Byron E. Anderson, Ph. D.

CURRICULUM VITAE

Byron E. Anderson

Date of Birth: December 30, 1941; Hammond, Indiana

Education:

B.A.	1963	Kalamazoo College, Kalamazoo, Michigan; Chemistry and Biology
Ph.D.	1968	University of Michigan, Ann Arbor, Michigan; Biochemistry; also student at John Hopkins University, Baltimore, Maryland 1966-1968. Thesis Advisor: Dr. Saul Roseman

Positions Held:

Postdoctoral Fellow - 1968-1971, Columbia University, College of Physicians and Surgeons, New York, New York. Advisor: Dr.Elvin A. Kabat.

Assistant Professor, Departments of Biochemistry, and Otolaryngology and Maxillofacial Surgery, Northwestern University Medical School, 1971-1977.

Associate Professor, Departments of Biochemistry, and Otolaryngology and Maxillofacial Surgery, Northwestern University Medical School, 1977-1982.

Associate Professor, Department of Molecular Biology and Biochemistry Program 1982-1984.

Professor, Departments of Molecular Biology, and Otolaryngology and Head and Neck Surgery, Northwestern University Medical School, 1984-1989.

Professor, Departments of Cell and Molecular Biology, and Otolaryngology and Head and Neck Surgery, Northwestern University Medical School, 1989-2002.

Professor, Department of Urology, 1995 - 2002.

Professor Emeritus,2002 - present

Member, Cancer Center, 1975 -

Member, Arthritis Research Program, 1976 - ; Multipurpose Arthritis Center, 1983 -

Member, Tumor Cell Biology Program, 1979 -

Member, Interdisciplinary Program in Molecular, Cellular and Integrative Biomedical Sciences, 1983 - 1990.

Member, Molecular and Cellular Biology Training Program, 1983 -
Member, Medical Scientist Training Program, 1985 -

Member, Biotechnology Training Program, 1993 -

Honors and Fellowships:

1974-1979, Research Career Development Awardee of NIH (NIAMDD)

1973-1974, Senior Investigator of the Arthritis Foundation

1968-1971, Postdoctoral Fellow of the Helen Hay Whitney Foundation and the National Cystic Fibrosis Research Foundation

1963-1968, United States Public Health Service Trainee

1963, summer National Science Foundation Undergraduate Trainee

Professional Societies and Activities:

American Association for the Advancement of Science
American Society of Biochemistry and Molecular Biology
American Association of Immunologists
International Society for Oncodevelopmental Biology and Medicine
Society for Complex Carbohydrates
American College of Rheumatology
International Society for Artificial Organs
The Protein Society
Advisor or Consultant to a number of biotechnology companies: Sangtec Medical, Fresenius, Abbott Labs., Chugai

Pharm. LTD, Vivex Therapeutics,Inc., Carbohydrates Int., Toagosei Pharm.

Co-founder and director of R&D - Immtech Int. Inc. 1984 - 2001

Co-founder and President - Septech, Inc. 2002 - present

Co-founder and President - Bio Science Int. 2002 - present

Students:

Celia Kaye, Ph.D.,	1976
Linda Hanson, M.S.,	1976
Margaret Steffes, M.S.,	1977
Lorraine Gill, Ph.D.,	1977
Tod Sloan, Ph.D.	1979
John Jay Weiss, M.S.,	1979
Lyman Davis, M.S.,	1980
Mark Pankow, M.S.,	1983
Lyman Davis, Ph.D.	1984
Ruth Entiwstle, Ph.D.,	1986
John Jay Weiss, Ph.D.,	1986
Mark Pankow, Ph.D.,	1988
Michael Baumann, Ph.D.,	1990
Michael Shields, Ph.D.,	1991
John J. Kresl, Ph.D.,	1992
Marilyn Brown, Ph.D.,	1992
Winnie Pao, M.S.	1993
NaMi Cho, M.S.,	1994
Joseph Orlando, M.S.,	1994
Sanjiv Gupta, M.S.,	1994
Katherine Worthington, Ph.D.,	1994
Jai Syn, M.S.,	1995
Cristopher Olenec, M.S.	1995
Lora Tucker-Garcia, M.S.	1995
Uri Ratner, M.S.	1996
Christina Stadler, M.S.	1996
Lora Kastrup, M.S.	1996
Marsha Yoder, M.S.	1997
Jasbir Kindra, M.S.	1997
Sharon Doering, M.S.	1997
Clara Smith, M.S.	1998
Ethan Buckley, M.S.	1999
Scott Rosenblum, M.S.	2000
Nancy Sullivan, M.S.	2000
Jeffery Black, M.S.	2000

Trainees:

Postdoctoral Fellows:

Deepika Paul	Mario Venegas
Samar Maklouf	Thomas Rohr
Jitendra Verma	Michio Tanaka
Shiva Motameni	Stewart Nelson
Lyman Davis	Ramendra Pandey
Bernard Kubak	Donna Bishop

Residents in Rheumatology:

Mike Repice Albert Iammartino
Charlotte Harris William Liu
Cynthia Gustafson

Residents in Otolaryngology:

Deyan Popovic Susan Lyon

Medical Students:

James Carr Robert Sobut Denise Mistro Susan Roth
Thomas Lucke Sarah Keesara Ragu Thunga Thomas Walsh
Thomas Henthorn Paul Lyon Martin Radvany Ateet Shah
Tapubrata Ghosh Annette Barns James Misak Ripal Ghandi
Roberta Gausas Reeva Shulruf Samir Taneja
William Walsh Kyle ver Steeg Sylvester Black

Biotechnology Program Students:

Winnie Pao	Cristina Stadler	Marcia Yoder
Ayano Takamoto	Christopher Olenec	Jasbir Kindra
Lourdes Bermejo	Jai Syn	Joseph Orlando
Uri Ratner	Nami Cho	Lora Kastrup
Sanjiv Gupta	Sharon Doering	Clara Smith
Ethan Buckley	Scott Rosenblum	Nancy Sullivan
Jeffrey Black		

Undergraduate Students:

Marlene Morrison	Loretta Liu	Jennifer Shaw
Daniel Fryxell	John Zachariah	Jason Roh
Michael Baumann	Denise Mistro	Andrew Hong
Ragu Thunga	Askok Kukadia	Kirsten Stadler
Martin Radvany	Gautham Reddy	SandyShaw
Andrew Radvany	Stephanie Wu	Katherine Chen
Daniel Roh	David Napochi	Thomas Kim

Students, Thesis, Present Position

Celia Kaye, M.D., Ph.D.
Cystine Reduction and Transport in Cystinotic and Normal Fibroblasts
Ph.D., Biochemistry, 1985
Head, Genetics, U. of Texas, San Antonio

Linda Hanson, M.S.
Characteristics of the Antigenic Reactivities of Rheumatoid Arthritic Synovial Fibroblasts
M.S., Clinical Pathology, 1976

Lorraine Gill, Ph.D.
Characterization of Reactivities of Anti-Cellular Sera with Tumor Cells in Vitro
Ph.D., Biochemistry, 1977
Research Scientist, New England Nuclear

Margaret Lu Steffes, M.S.
Delineation and Partial Characterization of an Associated Rhematoid Arthritic Reactive Antigen
M.S. Biochemsitry, 1977
Senior Manager, Abbott Labs

Tod B. Sloan, M.D., Ph.D.
A Study of Synovial Cell Antigens
Ph.D., Biochemsitry, 1978
Associate Professor, Dept. Anesthesiology, U. of Texas, San Antonio

John Jay Weiss, M.S., Ph.D.
Studies on the Platelet Derived Connective Tissue Activating Peptide
M.S., Clinical Pathology, 1979
Program Director in Allergy, Diagnostic Products Corp.

Lyman E. Davis, M.S., Ph.D.
Immunoassays for Fibronectin
M.S., Clinical Pathology, 1980
Senior Director, Takeda Abbott

Mark L. Pankow, M.S., Ph.D.
Immunochemical Staining of Cancer Tissues
M.S., Clinical Pathology, 1983
Director of R&D, Parke DeWatt Labs

Students, Thesis, Present Position - continued

Lyman E. Davis, M.S., Ph.D.
Specificities of Antibodies to a Carbodiimide and to the Amino-Terminus of a Platelet Mitogen
Ph.D., Tumor Cell Biology, 1984
Senior Director, Takeda Abbott

Ruth A. Entwistle, Ph.D.
A Kinetic Study of Fibronectin and the C1q Component of Complement Ph.D.,
Tumor Cell Biology, 1986
Research Associate, Washington U. School of Medicine

Mark L. Pankow, M.S., Ph.D.
Development of an Assay for Detection of Galactose Terminated Glycoprotiens in Biologic Fluids
Ph.D., Tumor Cell Biology, 1988
Director of R&D, Parke DeWitt Labs.

John Jay Weiss, M.S., Ph.D.
Studies of Complement Mediated Interactions Between Fibronectin and Immune Complexes
Ph.D., Tumor Cell Biology, 1989
Program Director in Allergy, Diagnostic Products Corp.

Michael A. Baumann, Ph.D.
Synthetic Peptides with Immune Complex Binding Activity
Ph.D., Tumor Cell Biology, 1990
Research Scientist, Abbott Labs.

Michael J. Shields, Ph.D.
Characterization and Use of Monoclonal and Polyclonal Antibodies
Directed Against C-Reactive Protein in the Fluid and Solid Phase
Detection of Altered Forms of C-Reactive Protein in Humans
Ph.D., Molecular Biology, 1991
Postdoctoral Fellow, National Institutes of Health

Students, Thesis, Present Position - continued

John Joseph Kresl, Ph.D., M.D.
Evaluation of Native Modified Human C-Reactive Protein Interconversion: Possible Role for Modified C-Reactive Proteins in Tumor Inhibition
Ph.D., Tumor Cell Biology, 1992, MD, 1993
Staff Physician, Radiation Oncology, St. Mary's Hospital,Phoenix,

Marilyn R. Brown, Ph.D.
Receptor-Ligand Interactions Between Serum Amyloid P Component
and Model Soluble Immune Complexes
Ph.D., Tumor Cell Biology, 1993
Research Scientist, Baxter Labs.

Winnie Pao, M.S.
Autoantibodies in Colon Cancer
M.S., Biotechnology Program, 1993
Research Associate, Division of Transplantation, NUMS

Katherine Worthington, Ph.D.
Design of Peptides Binding to Beta-2-microglobulin and Immunoglobulin G
Ph.D., Tumor Cell Biology, 1994
Research Analyst, Research Corp. Technologies

NaMi Cho, M.S.
Binding of IgG Aggregates to CBP2-Amino Link
M.S., Biotechnology Program, 1994
Postdoctoral Fellow, Boston University

Joseph Orlando, M.S.
CBP2 Binding of Aggregated-IgG
M.S., Biotechnology Program, 1994
Graduate Student, Dept. of Microbiology-Immunology,
Wake Forest University, Bowman Gray School of Medicine

Sanjiv Gupta, M.S.
Detection of a Transferrin Isoform Associated with Alcohol Consumption
M.S., Biotechnology Program, 1994
Medical Student, University of Indiana

Students, Thesis, Present Position - continued

Jai Syn, M.S.
Specificities of Binding of Lectins to an Aromatic Carbohydrate Mimetic Library
M.S., Biotechnology Program, 1995
Research Associate, Abbott Labs.

Christopher Olenec, M.S.
Binding of Proteins to a Secondary Structure Limited Peptide Library
M.S. Biotechnology Program, 1995.
U.S. Commerce Dept. on FDA Regulations, Washington, D.C.

Lora Tucker-Garcia, M.S.
Carbohydrate Deficient Glycoproteins: Significance in Carbohydrate Deficient Glycoprotein Syndrome, Alcoholism, and Fetal Alcohol Syndrome
M.S., Biotechnology Program, 1996
Research Associate, Abbott Labs.

Uri Ratner
Characterization of Xenoreactive Antibodies Important in Transplant Rejection
M.S. Biotechnology program, 1996
Research Analyst, Venture One

Cristina Stadler
Complement Inhibition by Peptides in Hyperacute Rejection Reactions
M.S., Biotechnology Program, 1996
Division Manager, Baxter Labs.

Lora Kastrup
Study of Vaginal Fluid Oligosaccharides in UTIs
M.S., Biotechnology Program, 1996
Research Associate, Baxter Healthcare Corporation

Jasbir Kindra, M.S., LL.D.
Complement Inhibition Through the Use of Diaromatic Peptides to Allow Xenotransplantation
M.S., Biotechnology Program, 1997
Patent Attorney, Green Bay, WI

Students, Thesis, Present Position

Marcia Yoder, M.S.
Studies on Complement Inhibition by a Diaromatic Peptide, Tryptophan-Tyrosine
M.S., Biotechnology Program, 1997
Research Associate, Eli Lilly Co.

Sharon Doering, M.S.
Avian IgY antibodies - Use in Xenotransplantation
M.S., Biotechnology Program, 1997
Biotechnology Analyst, Madison Securities

Clara Smith, M.S.
Cross-Linked Avian IgY and Human IgG as Potential Inhibitors of Rejection in Xenotransplantation
M.S., Biotechnology Program, 1998
Research Scientist, Glaxo Welcome

Committee Service:

Research Committee, Medical and Dental Schools
Sigma XI Symposia
Medical Applicant Interview
Cancer Center Library
General Services Committee of Faculty Senate
Faculty Senate
Biochemistry Workshop
Departmental Outside Speaker, Equipment, Library
Graduate Affairs Committee
VA Research Development Committee
Executive Committee of the Biochemistry Program
Cancer Center Equipment
Cancer Focus
Medical Admissions
Tumor Cell Biology Program
Comprehenisve Examination
Pre-Thesis and Thesis Committees for 33 students
University Research Grants Committee
General Faculty Committee
Graduate Professional Education of the Physician
Task Force on the Future of the Medical Library
Microbiology Department Review Committee
Medical Senate Council
Medical Council
Medical Library Long-Range Planning Committee
Program Review of Microbiology-Immunology
Program Review of Physical Therapy Curriculum
Research Program Committee on Immunobiology
Intellectual Property Committee
Medical School Urology Chair Search Committee
Medical School Division Making Course Development Committee
Medical School Research Council
Biotechnology Program: Executive Committee, Admissions,
Advisory Board
Honors Program in Medical Education
Program Review Committee - Mol. Pharm. & Biochem.

Committee Service 1984 -
University:

General Faculty Committee, 1982-1985
Subcommittee on Benefits
Chair, GFC/Trustees Meeting, 1983, 1984
Intellectual Property Committee, 1989-1996
University Research Grants Committee, 1973 - 1976; 1995 -
Biotechnology Program:Executive Committee, Admissions, Advisory Board, 1992 -

Medical School:

Medical Senate Council, 1984-1988
General Profession Education of the Physician Committee
Chair, Subcommittee on Faculty Involvement, 1985-1986
Research Long-Range Planning in Immunobiology, 1985-1986
Chair, Research Program Committee on Immunobiology, 1985- 1990
Medical School Admissions, 1972 -
Honors Program in Medical Education, 1996 -

Departmental:

Appointments, Promotion, Tenure
Graduate Affairs
Course/Exam/Certificate - Chair
Promotions - Chair
Library
Recruitment - Chair

Teaching Service:

1. Medical, Biochemistry, laboratory section
   Subjects: Statistics, acid-base balance, nutrition, automated analyses, topics in immunology and autoimmune diseases

2. Medical, Biochemistry, lecture
   Topics: glycogen metabolism
   mucopolysaccharides, saccharide interconversions
   glycoproteins
   hexose monophosphate pathway
   post-ribosomal processing of proteins

3. Medical, biochemistry, lecture and independent study of immunology with students with proficiency in biochemistry

4. Medical, immunology, lectures on antibody structure
   and antibody - antigen interactions

5. Medical, basic oncology, lectures on biological membranes

6. Graduate, survey course in immunology

7. Graduate, lecture and seminar on biological membrane
   topics

8. Graduate, lectures in immunlology on antibody structure, antigenic determinants, antibody-antigen interactions, antibody functions

9. Graduate, survey courses in biochemistry,
   Topics: carbohydrate sturcture, analysis;
   glycoprotein and glycolipid stucture, functions and metabolism; lipopolysaccharide
   structure and synthesis; biological membranes; muscle structure
   protein structure
   protein - ligand binding

10. Graduate, basic oncology, lectures on biologic membranes

11. Graduate, survey course in immunochemistry

12. Post-Graduate, lecture on inter-relationships of comple-
ment, fibrinolytic and kinin systems

LECTURES, MEDICAL BIOCHEMISTRY

1. Glycogen metabolism and regulation
2. Structural complementarily, amplification of metabolic response, disorders in glycogen metabolism
3. Galactose, glucuronic acid metabolism, mucopolysaccha-rides, saccharide interconversions, hexose monophosphate shunt
4. Post-ribosomal modification of proteins, protein polymorphisims and disease consequences
5. Fatty acid synthesis and degradation
6. Lipid structure and function
7. Phospholipid structure and function

BASIC ONCOLOGY:

1. Structural features of biologic membranes
2. Tumor cell membranes, tumor-associated antigens

GRADUATE BIOCHEMISTRY:

1. Stereochemistry of carbohydrates
2. Conformations of carbohydrates
3. Chemical reactivity and conformation
4. Methodology for structural determinations of carbohydrate sequences
5. Types and characteristics of complex carbohydrates
6. Methods applied to analysis of complex carbohydrates
7. Synthesis of complex carbohydrates
8. Functions of complex carbohydrates
9. Proteins - primary and secondary structure
10. Proteins - Tertiary and quaternary structure
11. Protein-protein and protein-ligand interactions
12. Proteins - structural predictions
13. Enzyme kinetics
14. Enzyme mechanisms

15. Enzymes - Types and regulation
16. Membrane structure and function
17. DNA and RNA structures
18. Principles that determine polynucleotide structure
19. Protein - polynucleotide interactions

LECTURES, DENTAL BIOCHEMISTRY

1. Glycogen metabolism and regulation of blood glucose
2. Structure and function of phospholipids, membrane structure
3. Fatty acid synthesis and degradation
4. Interrelationships and regulation of metabolic pathways

IMMUNOCHEMISTRY:

1. Heterogeneity of the antibody response; classes, subclasses, allo- and idiotypes of Igs

2. Structural features of Igs; discussion of homologies and deduction of structure from amino acid sequence data

3. Effector functions of the Igs

4. Three dimensional structure of Igs and quaternary interactions of domains

5. Antibody combining site constructs

6. Antibody combining site interactions with haptens; serologic specificities

7. Measurement, interpretation and quantitations of antibody-antigen interactions

8. Kinetic analyses of antibody-hapten interactions

9. Radio- and enzyme-immunoassays

10. Parameters of antigenicity and immunogenicity

CANCER PHARMACOLOGY:

1. Progression and selection of tumor cell populations; changes in cell surface composition and interactions with metastases

2. Tumor-associated antigens

RESIDENT AND STAFF LECTURES:

Otolaryngology: lectures on overview of tumor immunology

Rheumatology:

1. Synovial cell metabolism and antigen, genetic analysis of antigen expression

2. Protein Polymorphisms: types, effects on protein function, contribution to disease processes

Subjects taught in Medical Biochemistry Laboratory:

1. Principles and methods of automated analyses

2. Acid-base regulation and imbalances

3. Statistical methods and analyses of clinical data

4. Dietary calculations of protein, carbohydrate, lipid, caloric intake and nitrogen balance

5. Presentations, analysis, critique of primary clinical and basic research papers

Graduate Thesis Studies

Abstract: Kundig, W., Kundig, F. Dodyk, Anderson, B. and Roseman, S., "Galactose-6-Phosphate Synthesis by a Phosphotransferase System," Fed. Proc., 24, 658, 1965.

Kundig, W., Kundig, F. Dodyk, Anderson, B. and Roseman, S., "Restoration of Transport of Glycosides in Escherichia coli by a Component of a Phosphotransferase System," J. Biol. Chem., 241, 3243-3245, 1966.

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PATENT APPLICATIONS

1. Binding of immune complexes by modified forms of c-reactive protein.
   Applicants: Northwestern University and
   Rush - Presbyterian - St. Luke's Medical Center
   Inventors: Potempa and Anderson
   U.S. application Serial No. 07/582,884,
   Filed: October 3, 1990
   Refiled: 08/271,137; July 6, 1994 - 2545/73
   Status: Issued: January 14, 1997; US Letter Patent No. 5,593,897
   PCT application number PCT/US89/01247
   Filed March 31, 1989 (2545/11)
   Status: Nationalized

   Australian application no. 34485/89
   Filed: March 31, 1989 (2545/24)
   Status: Issued, 6/11/93; Serial #633488

   Japanese application No. 1-504716
   Filed: March 31, 1989 (2545/26)
   Status: Pending, awaiting examination

   Canadian application No. 595,543
   Filed April 3, 1989 (2545/10)
   Status: Pending, no action yet

   EPO application No. 89904944.9
   Filed March 31, 1989 (2545/25)

2. A synthetic peptide and its uses
   Applicant: Northwestern University
   Inventors: Baumann and Anderson
   U.S. application Serial No. 07/598,416
   Filed: October 16, 1990 (2545/28)
   Status: Issued November 15, 1994
   Patent Number 5,364,930

   PCT application number PCT/US91/07581
   Filed: October 9, 1991
   Status: Pending

3. Binding of aggregated immunoglobulin or immune complexes by serum amyloid P component
Applicant: Northwestern University
Inventors: Anderson and Brown
U.S. application Serial No. 07/672,526

Filed: March 19, 1991 (2545/21)
Status: Issued 6/22/93, P-5,221,628

4. Immunoassay for glycosylated proteins employing antibody to reductively glycosylated amino acids
Applicant: Northwestern University
Inventors: Davis and Anderson
U.S. application Serial No. 07/397,781 (2545/5)
08/068,525 (5/27/93) (2545/51)
08/151,073 (11/12/93) (2545/52)
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Status: Nationalized

EPO application no. 90913261.5
Filed: August 17, 1990 (2545/37)
Status: Granted

Japanese application no. 2-512516
Filed: August 17, 1990 (2545/38)
Status: Pending, awaiting examination

5. Immunoassay for detecting and monitoring alcoholics
Applicants: Northwestern University and Immtech Int. Inc.
Inventors: Makhlouf, Pankow, Anderson and Bean
U.S. application Serial No. 07/765,169;
Filed: September 25, 1991 (2545/8)
08/272,852; July 08, 1994 (2545/74)
Status: granted

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Japanese application no. 5-506376
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Canadian application no. 2119651
Filed: 9/25/92 (2545/59), granted

Australian application no. 27577/92
Filed 9/23/92 (2545/58), granted

6. Methods of Treating Cancer Using Modified C-Reactive Protein
Applicants: Northwestern University and Immtech Int. Inc.
Inventors: Potempa, Kresl and Anderson
U.S. application Serial No. 07/874,263
Filed: April 24, 1992
Status: Issued December 12, 1995
US Letters Patent No. 5,474,904
PCT application no. PCT/US/03769
Filed: 4/22/92 (2545/50)
Status: Nationalized
EPO application no. 93910710.8, Filed: 4/22/93 (2545/81)

Japanese application no.5-518361, Filed: 4/22/93 (2545/80)

Canadian application no. 2432001, Filed: 4/22/93 (2545/80)

Australian application no. 41109/93,
Filed: 4/22/93 (2545/79)

7. Method of detecting cancer
Applicants: Northwestern University and Immtech Int. Inc.
Inventors: Anderson and Davis
U.S. application Serial No. 07/939,830
Filed: September 3, 1992 (2545/9)
Status: Issued December 27, 1994
Patent Number 5,376,531

Japanese application No. 4-293052
Filed: October 30, 1992 (2545/45)
Status: Pending

8. Methods of Imaging Cancer Cells Using Modified C-Reactive Protein
Applicant: Northwestern University
Inventors: Potempa, Kresl and Anderson
U.S. Application 149,663
Filed Nov. 9, 1993
Status: Issued December 12, 1995
US Letters Patent No. 5,474,904

9. CIP to Synthetic Clq Peptide Fragments - use in transplantation
Applicant: Northwestern University
Inventors: Baumann, Anderson and Fryer
Filed: April 6, 1996 to US patent office
Status: granted

PCT of same:
Application and Inventors: Baumann, Anderson and Fryer
Filed: April 4, 1997
Status: withdrawn

10. Method of Inhibiting Complement
Applicants and Inventors: Anderson and Fryer
Filed: April 14, 1997 to US patent office
Status: granted

11. Peptides Comprising Aromatic D-Amino Acids and Methods Of Use
Inventor: Byron E. Anderson
US patent filed July 3, 2002
PCT application filed July 3, 2003

libraries of longer or more diverse peptides should they be required for any given application.

We have expanded the applications of our peptide library approach by modifying the synthesis procedure to incorporate cleavable linkers on each bead. After exposure to the cleaving agent, such beads can then release a portion of their peptides into solution for biological assay while still retaining sufficient peptides on the beads for subsequent structure determination.

The one-bead, one-peptide concept and its applications discussed above demonstrate that this approach provides important new tools with which to search for specific ligands of potential diagnostic or therapeutic value. Such information should also enhance fundamental understanding of interactions between ligands and acceptor molecules. □

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Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery

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EXISTING methods for the synthesis and screening of large numbers of peptides are limited by their inability to generate and screen the requisite number (millions) of individual peptides[1–4] and/or their inability to generate unmodified free peptides in quantities able to interact in solution[4–8]. We have circumvented these limitations by developing synthetic peptide combinatorial libraries composed of mixtures of free peptides in quantities which can be used directly in virtually all existing assay systems. The screening of these heterogeneous libraries, along with an iterative selection and synthesis process, permits the systematic identification of optimal peptide ligands. Starting with a library composed of more than 34 million hexa-peptides, we present here the precise identification of an antigenic determinant recognized by a monoclonal antibody as well as the straightforward development of new potent antimicrobial peptides.

The initial synthetic peptide combinatorial library (SPCL) prepared and used in this work consisted of six-residue peptide sequences with acetylated N terminals and amidated C terminals. The first two positions in each peptide were individually and specifically defined, whereas the last four positions consisted of equimolar mixtures of 18 of the 20 natural L-amino acids (for ease of synthesis, cysteine and tryptophan were omitted in this initial library). Such libraries can be generally repre by the sequence Ac-O_1O_2XXXX-NH_2 (where Ac rep acetyl) (see legend to Fig. 1).

Using a competitive enzyme-linked immunosorbent (ELISA), each of the 324 different peptide mixtures of the (Ac-O_1O_2XXXX-NH_2) was assayed to determine its ab inhibit the interaction of a monoclonal antibody with a 13-residue peptide (Ac-YPYDVPDYASLRS-NH_2, singl amino-acid code). Of the 324 peptide mixtures examine 1), Ac-DVXXXX-NH_2 caused the greatest inhibition o body binding (Table 1). Twenty new peptide mixtures we synthesized in which the third position of the peptide r Ac-DVXXXX-NH_2 was defined (Ac-DVOXXX-NH_2 tophan now included in the X positions). Each new p mixture contained 6,859 (19^3) individual peptides (137 total). The most effective inhibiting peptide mixture w DVPXXX-NH_2 (50% inhibitory concentration, IC_{50} = Table 1*b*). The above iterative process, which reduces th ber of peptide sequences by 20-fold each time it is re was then carried out for the remaining three positions 1, *c–e*). It should be noted that on defining the fifth p (Ac-DVPDOX-NH_2, Table 1*d*), the IC_{50} found fo DVPDYX-NH_2 (0.38 μM) was at least 3,500-fold lowe any of the other 19 peptide mixtures. Also, the peptide m Ac-DVPDXX-NH_2 and Ac-DVPXXX-NH_2 had IC_{50} lower than all of the peptide mixtures with the fifth p defined, with the exception of Ac-DVPDYX-NH_2. This

TABLE 1 Identification of the antigenic determinant recognized b clonal antibody 19B10

Peptide mixture	IC_{50} (μM)	Peptide
(*a*)		(*e*)
Ac-DVXXXX-NH_2	250	Ac-DVPDYA-NH_2
Ac-DIXXXX-NH_2	318	Ac-DVPDYS-NH_2
Ac-DMXXXX-NH_2	752	**Ac-DVPDYX-NH_2**
Ac-DLXXXX-NH_2	>1,400	Ac-DVPDYC-NH_2
		Ac-DVPDYV-NH_2
(*b*)		Ac-DVPDYT-NH_2
Ac-DVPXXX-NH_2	41	Ac-DVPDYG-NH_2
Ac-DVEXXX-NH_2	146	Ac-DVPDYE-NH_2
Ac-DVQXXX-NH_2	215	Ac-DVPDYI-NH_2
Ac-DVXXXX-NH_2	**250**	Ac-DVPDYM-NH_2
Ac-DVMXXX-NH_2	451	Ac-DVPDYQ-NH_2
Ac-DVRXXX-NH_2	906	Ac-DVPDYH-NH_2
Ac-DVAXXX-NH_2	1,107	Ac-DVPDYL-NH_2
Ac-DVCXXX-NH_2	>1,400	Ac-DVPDYR-NH_2
		Ac-DVPDYF-NH_2
(*c*)		Ac-DVPDYN-NH_2
Ac-DVPDXX-NH_2	4.4	Ac-DVPDYK-NH_2
Ac-DVPXXX-NH_2	**41**	Ac-DVPDYY-NH_2
Ac-DVPAXX-NH_2	>1,400	Ac-DVPDYP-NH_2
		Ac-DVPDYW-NH_2
(*d*)		Ac-DVPDYD-NH_2
Ac-DVPDYX-NH_2	0.38	
Ac-DVPDXX-NH_2	**4.4**	
Ac-DVPDAX-NH_2	>1,400	

The IC_{50}s of the most effective inhibitory peptide mixtures obt each iterative step are illustrated for: *a*, peptide mixtures from th screening of the SPCL; *b*, the third position defined (Ac-DVOXXX-NH fourth position defined (Ac-DVPOXX-NH_2); *d*, the fifth position defi DVPDOX-NH_2); and *e*, the sixth position defined (Ac-DVPDYO-NH_2). of the peptide mixture derived from the previous iterative step is for comparison. Peptide mixtures were assayed by competitive ELI Fig. 1). The concentration of each peptide mixture necessary to inhi of the antibody binding to the control peptide on the plate was obta serial dilutions of the peptide mixture. The IC_{50}s were calculated u software GRAPHPAD (ISI, San Diego). The four-step iterative screer synthesis process takes approximately 4 weeks. This time frame depending on the assay being used and the number of cases moved at each iterative step.

illustrates the importance of the presence of specific peptide sequences in each peptide mixture. Among the 20 peptides in which the sixth position was defined, Ac-DVPDYA-NH_2 had the lowest IC_{50} (0.03 μM; Table 1*e*). This sequence exactly matches the antigenic determinant found in earlier studies to be recognized by this monoclonal antibody[2,9,10]. With other screening library procedures[6–8], such precise sequence determination, or the identification of different sequences with affinities equal to or exceeding existing sequences, was not accomplished. The results presented here confirm our earlier work in which individual peptides[8–11] or chemically synthesized heterogeneous peptide mixtures[12,13] were used to establish that each position in a linear antigenic determinant has a specific, quantifiable rank order of importance. The use of this SPCL permits the ready determination of the specific peptide sequence that bound to this antibody out of a total of 34,012,224 possible hexapeptides. Note that no information about the sequence of the antigen or antibody is required to carry out determinations of this kind.

The development of new, potentially useful therapeutic peptides[14–19] requires the synthesis and screening of hundreds to thousands of analogues of an original, often serendipitously discovered active sequence. The potential of SPCLs for the development of new antimicrobial peptides against *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria), and the yeast *Candida albicans* was examined in microdilution assays using the same set of 324 peptide mixtures making up the Ac-O_1O_2XXXX-NH_2 peptide library used above. Although many antimicrobial lead peptide sequences derived from this SPCL have been followed in detail (manuscript in preparation), a single example (Ac-RRXXXX-NH_2) found effective against the above microorganisms is presented here. Positions three to six of Ac-RRXXXX-NH_2 were defined using the iterative process described above (the data for *S. aureus* are shown in Table 2). The minimum inhibitory concentrations (MIC) of the 20 individual peptides obtained on defining the sixth position of Ac-RRWWCX-NH_2, as well as the C-terminal amide form of the naturally occurring antimicrobial peptide magainin, are shown in Table 3. The hexa-peptide Ac-RRWWCR-NH_2 was the most active of these sets. Its MIC against *S. aureus* was 3.2–6.5 μg ml^{-1}. Preliminary data indicate that this peptide is bactericidal. The haemolytic activity of Ac-RRWWCR-NH_2 was less than 0.2% at 500 μg ml^{-1}. It is noteworthy that the antistaphylococcal activities of 17 of the 20 sequences were greater than magainin[15].

The use of SPCLs has been illustrated here for both the precise identification of a linear antigenic determinant recognized by a monoclonal antibody and for the development of new, highly effective antimicrobial peptides. A number of other libraries,

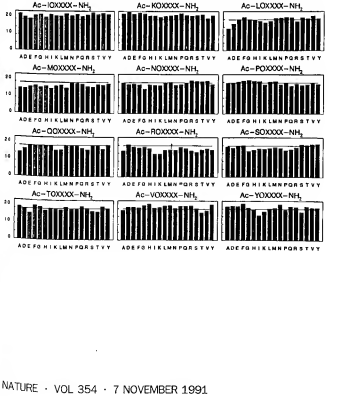

FIG. 1 Initial screening of the SPCL (Ac-O_1O_2XXXX-NH_2) for ability to inhibit the binding of monoclonal antibody 19E10. Each of the 324 peptide mixtures of the SPCL was assayed by competitive ELISA for its ability to inhibit the binding of monoclonal antibody 19E10 (ref. 20) to the plate-adsorbed peptide Ac-YPYDVPDYASLRS-NH_2. The individual bar graphs are segregated by first amino acid (O_1), with the individual bars in each graph representing the 18 individual amino acids making up the second position (O_2). The *y*-axis represents optical density (OD) at 492 nm. The horizontal line in each bar graph represents the average OD of the 324 peptide mixtures. O_1 and O_2 are specific individual amino acids; that is O_1O_2 = AA, AD, AE, and so on through to YV, YY, for a total of 324 combinations at positions O_1 and O_2 (18^2). Each X position represents an equimolar mixture of the 18 amino acids A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, Y for a total of 104,976 combinations (18^3). Each of the 324 different peptide mixtures consists of 104,976 individual hexamers, which represent 34,012,224 peptides in total (324 × 104,976). Assuming an average relative molecular mass for Ac-O_1O_2XXXX-NH_2 of 785, then a mixture of 104,976 peptides (18^4) at a total final concentration of 1.0 mg ml^{-1} yields a concentration of every peptide in each mixture of 9.53 ng ml^{-1} (12.1 nmol l^{-1}).

METHODS. The synthetic peptide library was prepared using methylbenzhydrylamine (MBHA) polystyrene resin and standard *t*-Boc chemistry in combination with simultaneous multiple peptide synthesis (SMPS[8]). A divide, couple and recombine (DCR) process was used to synthesize the XXXX-peptide resin. This process assures equimolarity of the peptides on the resin. Briefly, 18 porous polypropylene packets, each containing 4.65 mmol (5.00 g) of MBHA resin, were coupled to each of the protected *N*-α-*t*-Boc amino acids of interest. All coupling reactions proceeded to completion (>99.5%), as assessed by Gisin's picric acid[21] or Kaiser's ninhydrin tests[22]. The resulting resins from each packet were then combined and thoroughly mixed. This resin mixture was separated into 18 portions of equal weight which were placed into porous polypropylene packets, followed by *N*-α-*t*-Boc protecting group removal and neutralization of the resulting amine TFA salts. The resin packets were then reacted with solutions of the individual activated amino acids to yield the 324 dipeptide combinations (18^2). The above DCR process was repeated twice more, yielding a final mixture of 104,976 protected tetra-peptide resins (18^4). This XXXX-resin was divided into 324 aliquots (150 mg each) and placed in numbered, porous polypropylene packets. Synthesis of the next two defined positions was carried out by SMPS. The peptide mixtures were deprotected and cleaved from their respective resins using low-high hydrogen fluoride (HF)[23] as described for individual peptides earlier[17,24] in a multiple HF cleavage apparatus (Multiple Peptide Systems, San Diego, California). Extraction of the individual peptide mixtures was carried out with H_2O. The time frames for synthesis, and the amounts of each peptide mixture obtained, are the same as described earlier for this number of individual peptides[3]. The competitive ELISA used is a modification of the direct ELISA technique described previously[10]. It differs only in the antibody addition step in which 25 μl each peptide mixture of the SPCL was added, with a fixed dilution of antibody 19E10 (25 μl per well).

TABLE 2 Antimicrobial activity against *S. aureus* obtained in the iterative process

Peptide mixture	IC_{50} ($\mu g\ ml^{-1}$)	Peptide mixture	IC_{50} ($\mu g\ ml^{-1}$)
(*a*)		(*c*)	
Ac-RRWXXX-NH_2	216	Ac-RRWWCX-NH_2	8.7
Ac-RRYXXX-NH_2	239	Ac-RRWWWX-NH_2	9.9
Ac-RRRXXX-NH_2	275	Ac-RRWWRX-NH_2	9.9
Ac-RRHXXX-NH_2	286	Ac-RRWWFX-NH_2	12
Ac-RRCXXX-NH_2	338	Ac-RRWWIX-NH_2	14
Ac-RRXXXX-NH_2	**450**	**Ac-RRWWXX-NH_2**	**32**
(*b*)		(*d*)	
Ac-RRWWXX-NH_2	36	Ac-RRWWCR-NH_2	3.4
Ac-RRWFXX-NH_2	58	Ac-RRWWCW-NH_2	4.1
Ac-RRWRXX-NH_2	77	Ac-RRWWCV-NH_2	4.9
Ac-RRWCXX-NH_2	88	Ac-RRWWCY-NH_2	5.4
Ac-RRWLXX-NH_2	178	Ac-RRWWCK-NH_2	5.5
Ac-RRWXXX-NH_2	**273**	**Ac-RRWWCX-NH_2**	**8.7**

The five lowest IC_{50}s obtained are illustrated for the peptide mixtures on defining: *a*, the third position (Ac-RROXXX-NH_2); *b*, the fourth position (Ac-RRWOXX-NH_2); *c*, the fifth position (Ac-RRWWOX-NH_2); and *d*, the sixth position (Ac-RRWWCO-NH_2). The IC_{50} of the peptide mixture derived from the previous iterative step is in bold for comparison. The antimicrobial activity of each peptide mixture against *S. aureus* ATCC 29213 was determined as described earlier[28]. Briefly, in 96-well tissue culture plates, peptide mixtures were added to the bacterial suspension ($1–5 \times 10^5$ colony-forming units ml^{-1}) at concentrations derived from serial twofold dilutions ranging from 1.5 mg ml^{-1} to 2.9 $\mu g\ ml^{-1}$. The plates were incubated overnight at 37 °C, and the growth determined at each concentration by the optical density at 620 nm. The relative per cent of growth found for each set of peptide mixtures was consistent in three separate determinations. The IC_{50}s were then calculated using the software program GRAPHPAD (ISI).

TABLE 3 Antimicrobial activity of Ac-RRWWCO-NH_2 against *S. aureus*

Sequence	MIC ($\mu g\ ml^{-1}$)	Sequence	MIC ($\mu g\ ml^{-1}$)
Ac-RRWWCR-NH_2	3.2–6.5	Ac-RRWWCA-NH_2	9–18
Ac-RRWWCV-NH_2	3.8–7.7	Ac-RRWWCP-NH_2	10–19
Ac-RRWWCW-NH_2	4.5–9.0	Ac-RRWWCM-NH_2	14–27
Ac-RRWWCY-NH_2	4.7–9.5	Ac-RRWWCL-NH_2	14–27
Ac-RRWWCK-NH_2	4.8–9.6	Ac-RRWWCI-NH_2	17–34
Ac-RRWWCT-NH_2	4.9–10	Ac-RRWWCF-NH_2	17–34
Ac-RRWWCH-NH_2	5.5–11	Ac-RRWWCC-NH_2	19–38
Ac-RRWWCQ-NH_2	5–12	**Magainin-II-NH_2**	**32–64**
Ac-RRWWCS-NH_2	7–14	Ac-RRWWCE-NH_2	>250
Ac-RRWWCX-NH_2	**7–14**	Ac-RRWWCG-NH_2	>500
Ac-RRWWCN-NH_2	8–16	Ac-RRWWCD-NH_2	>1,000

The MICs for the 20 peptides in which the sixth position is defined (Ac-RRWWCO-NH_2) are shown. The MIC is defined as the lowest concentration of peptide at which no growth is detected after 21 h incubation at 37 °C. The MICs of the previous peptide mixture and magainin-II are bold for comparison.

such as one composed entirely of D-amino acids, have been prepared which in total permit the systematic screening of hundreds of millions of peptides. A fundamental feature of SPCLs is that free peptides can be generated and used in solution in virtually all existing assay systems at a concentration of each peptide most applicable to the assay. This approach has also been successfully used in radio-receptor assays (opioid peptides) and plaque inhibition assays (human immunodeficiency virus (HIV-1) and herpes simplex virus (HSV). SPCLs, as described, greatly aid all areas of drug discovery and research involving peptides. □

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ERRATUM

Ancient oceans, ice sheets and the hydrological cycle on Mars

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IN this Article in the 15 August 1991 issue, an error in the *Natu* office led to the omission of a line from Table 3. In additio some corrections noted by the authors were not made befo publication. Corrected versions of the relevant passages appea below and in reprints.

TABLE 3 Possible sources of CO_2 related to catastrophic outflow an ocean formation on Mars

Source	CO_2 partial pressure (mbar)
North polar cap	~20
Massive volcanism	~100
Adsorbed on regolith: ocean basin	≤100
land	≤350
Groundwater	≤1,300
CO_2 clathrate	≤4,000

Fluvial history

In first paragraph:

The very high infiltration capacities of common martian surface rocks (lava flows and impact-brecciated regolith) would allow subsurface aquifers to be replenished easily, so that head differentials could be sustained and drive prolonged groundwater flow. The resulting sapping[31] would then produce the observed pattern of structurally controlled, low-density valley networks[24].

Although Noachian valleys are consistent with atmospheric

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Synthetic peptide combinatorial libraries (SPCLs): identification of the antigenic determinant of β-endorphin recognized by monoclonal antibody 3E7

(Bioactive peptide; competitive ELISA; receptor; drug discovery methods; iterative selection process)

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SUMMARY

The use of synthetic peptide combinatorial libraries (SPCLs), each composed of tens of millions of peptides, is described here for the identification of bioactive peptides. The identification of optimal peptide sequences is achieved through the screening of SPCLs in solution, each element of which is composed of more than 10^5 nonsupport-bound peptides in approximately equimolar representation, along with an iterative synthesis and screening process. Using an SPCL composed in total of 52 128 400 nonacetylated hexapeptides, along with an iterative selection process based on competitive ELISA, we identified the antigenic determinant of β-endorphin recognized by monoclonal antibody (mAb) 3E7. These results will be compared with the results found by others investigating mAb 3E7 using different peptide library approaches.

INTRODUCTION

The selectivity of biologically relevant receptor systems enables the activity of a peptide to be determined in the presence of large numbers of other inactive sequences. Bioactive peptides are typically active in the 10^{-8} to 10^{-10} M range, while present in an immensely complex milieu of other substances. This fact has long been exploited to find bioactive substances that have value as human therapeutics, but which are present in extremely low concentrations in plant, bacterial, amphibian, and mammalian tissues. A number of synthetic and recombinant phage approaches have been designed in an attempt to avoid the task of synthesizing and screening millions of individual peptides (reviewed by Birnbaum and Mosbach, 1992). All of these rely upon the inherent selectivity of biological receptor systems.

Synthetic peptide combinatorial libraries (SPCLs) are made up of nonsupport-bound mixtures of peptides covering all, or the majority of, the possible peptide sequences of a particular length. Each SPCL is prepared in quantities that can be used directly in existing solution assays (Houghten et al., 1991; 1992a,b,c; Pinilla et al., 1992; Houghten and Dooley, 1992). The screening of these heterogeneous libraries, along with an iterative selection process, permits the systematic identification of optimal peptide ligands using virtually any in vitro bioassay system. An example of the usefulness of this approach is described here, in which the antigenic determinant of β-endorphin recognized by mAb 3E7 is precisely identified.

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Abbreviations: aa, amino acid(s); ELISA, enzyme-linked immunoabsorbent assay; IC_{50}, inhibitory concentration of 50% mAb binding; mAb, monoclonal antibody; SPCL, synthetic peptide combinatorial library.

EXPERIMENTAL AND DISCUSSION

(a) Preparation of a six-residue, L-amino acid SPCL

The SPCL used for the current study consists of 6-aa peptide sequences having nonacetylated N terminals and

amidated C terminals. The first two aa in each peptide chain were individually and specifically defined, while the last four aa consisted of equimolar mixtures of 19 of the 20 natural L-aa. Cys was omitted from the mixture positions of the SPCL, but included in the defined positions. This library can be generally represented by the sequence $O_1O_2XXXX\text{-}NH_2$. O_1 and O_2 are specific individual aa [i.e., O_1O_2 = AA, AC, AD, etc., through YV, YW, YY, for a total of 400 combinations (20^2)]. Each X position represents an equimolar mixture of the 19 aa; four positions result in a total of 130 321 combinations (19^4). Therefore, each of the 400 different peptide mixtures that make up this library consists of 130 321 individual hexamers, which in total represent 52 321 400 peptides. Following the final cleavage from the resin, only extraction and lyophilization were necessary before use. Assuming that the average M_r of $O_1O_2XXXX\text{-}NH_2$ is 785, then a mixture of approximately 130 321 peptides at a total final concentration of 1.0 mg/ml yields an average concentration of 9.8 nM for individual peptides within each mixture.

(b) Identification of the antigenic determinant of β-endorphin

(1) Initial screening

Using competitive ELISA, each of the 400 peptide mixtures of the nonacetylated SPCL ($O_1O_2XXXX\text{-}NH_2$) was assayed at 2.5 mg/ml to determine its ability to inhibit the interaction of mAb 3E7 (Gramsch et al., 1983; Dandekar et al., 1985) with the antigenic peptide (β-endorphin, YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE-OH) adsorbed to the microtiter plate (Fig. 1). Of the 400 peptide mixtures examined, $YAXXXX\text{-}NH_2$ and $YGXXXX\text{-}NH_2$ were found to cause significant inhibition of mAb binding (i.e., >50%). The IC_{50} of $YGXXXX\text{-}NH_2$ was 2.9 μM, while the IC_{50} of $YAXXXX\text{-}NH_2$ was 10 μM.

(2) Iterative process

Following the above determination of the most effective peptide mixture from the initial screening of the peptide library, an iterative process was carried out in which the subsequent X positions of the peptide mixtures were individually defined with each of the 20 natural L aa. This process involves ranking, selecting, and reducing the number of peptide sequences while defining one more position at each step. Thus, 20 new peptide mixtures were synthesized in which the third position of the peptide mixture $YGXXXX\text{-}NH_2$ was defined (this is represented by the formula $YGOXXX\text{-}NH_2$) and assayed by competitive ELISA. Each peptide mixture now contained 6 859 (19^3) individual peptides. The two most effective inhibiting peptide mixtures were found to be $YGFXXX\text{-}NH_2$ (IC_{50} = 400 nM) and $YGGXXX\text{-}NH_2$ (IC_{50} = 1000 nM; Table IA). $YGHXXX\text{-}NH_2$ and $YGYXXX\text{-}NH_2$ were also found to effectively inhibit mAb binding (IC_{50} = 3000 nM), but they were not pursued at this stage. Upon defining the fourth position of $YGFXXX\text{-}NH_2$, $YGFLXX\text{-}NH_2$ and $YGFWXX\text{-}NH_2$ were found to significantly inhibit mAb binding (Table IB); the range from most effective (IC_{50} = 400 nM) to least effective (IC_{50} = 14 400 nM) was approximately 30-fold. In contrast, after assaying $YGGOXX\text{-}NH_2$ (Table IB), it was found that $YGGFXX\text{-}NH_2$ (IC_{50} = 48 nM) was more than 20-fold more effective than the next best peptide mixture ($YGGLXX\text{-}NH_2$), and 35 000-fold more effective than the least potent mixture, indicating the relative importance of the Phe at the fourth position of this peptide-mAb interaction.

At this point of the iterative process, we chose to further investigate only $YGGFXX\text{-}NH_2$ (IC_{50} = 48 nM) because of its tenfold higher inhibitory activity relative to $YGFLXX\text{-}NH_2$ (IC_{50} = 400 nM). Upon defining the fifth position ($YGGFOX\text{-}NH_2$), only $YGGFMX\text{-}NH_2$ (IC_{50} = 6.6 nM; Table IC), as well as $YGGFIX\text{-}NH_2$ and $YGGFLX\text{-}NH_2$, were found to significantly inhibit mAb binding when compared to the previous peptide mixture, $YGGFXX\text{-}NH_2$. Among the 20 peptides in which the sixth position was defined, $YGGFMT\text{-}NH_2$ had the lowest IC_{50} (3.2 nM; Table ID). The fact that the range of activities of the 18 defined peptides was only eightfold illustrates the relative redundancy of this position. The peptide $YGGFMT\text{-}NH_2$, found here to be the optimal peptide, exactly matches the first six aa of β-endorphin.

(c) Other peptide library approaches using mAb 3E7

Two other groups employing different peptide library approaches have also studied mAb 3E7. In the first of these studies, peptides were expressed on phage (Cwirla et al., 1990). After panning on mAb 3E7, 51 individual phage were recovered and sequenced. The deduced aa sequences were found to contain Tyr and Gly in the first and second positions, respectively. A variety of aa were found for the third position, whereas only aromatic (Phe or Trp) or aliphatic (Ile or Leu) residues were found at the fourth position. The fact that the correct residue at the fourth position, namely Phe, was found in less than 14% of the deduced aa sequences is at variance with the results found using the SPCL and iterative selection approach described here. Phe at the fourth position was found to be highly specific for mAb binding to peptides in solution (see section **b** above), and presumably the same is true of peptides expressed on phage. As noted by Cwirla and coworkers (1990), their failure to select strongly for Phe at position four may indicate poor

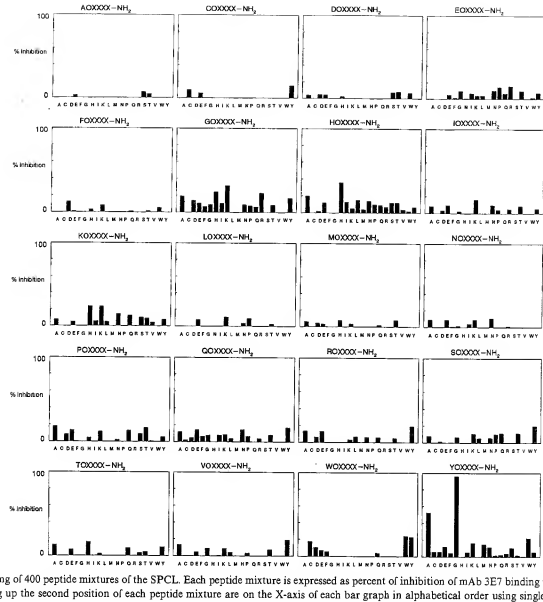

Fig. 1. Screening of 400 peptide mixtures of the SPCL. Each peptide mixture is expressed as percent of inhibition of mAb 3E7 binding to β-endorphin. The aa making up the second position of each peptide mixture are on the X-axis of each bar graph in alphabetical order using single letter aa code. **Methods: Peptide synthesis:** The 400 peptide mixtures making up the SPCL, as well as the peptide mixtures synthesized for the iterative steps, were prepared using methylbenzhydrylamine (MBHA) polystyrene resin and standard t-Boc chemistry in combination with simultaneous multiple peptide synthesis (SMPS; Houghten, 1985). Peptide mixture resins were prepared using a process termed divide, couple, and recombine [DCR] (Furka et al., 1991; Houghten et al., 1991). This process generates a single peptide on each individual resin bead. Briefly, 19 porous polypropylene packets, each containing 18.6 mmoles (20 g) of MBHA resin, were coupled to each of the protected N-α-t-Boc natural L-aa; Cys was excluded since its presence in the mixture positions would either require the constant presence of a reducing agent in the assay being used, or would result in ill-defined disulfide aggregates in the library. All coupling reactions proceeded to completion (>99.5%) as assessed by ninhydrin (Kaiser et al., 1970) or picric acid assay (Gisin, 1972). The resulting resins from each packet were then combined and thoroughly mixed. This resin mixture was divided into 19 portions of equal weight that were placed back into porous polypropylene packets, followed by N-α-t-Boc protecting group removal and neutralization. The resin packets were then reacted once again with solutions of the individual activated aa to yield the 361 dipeptide combinations (19^2). The above two cycles were repeated twice more, yielding a final mixture of 130 321 protected tetra-peptide resins (19^4). The above peptide resins (XXXX-resin) were divided into 400 aliquots and placed in numbered, porous, polypropylene packets. Synthesis of the next two defined positions was then carried out by SMPS. The total number of beads in the synthesis (approx. 1.5×10^8) vastly exceeds the total number of possible peptides (5.21×10^7); therefore, the library consists of all possible combinations of the aa used for a 6-aa peptide (thus the term 'combinatorial' in SPCL). The peptide mixtures were deprotected and cleaved from their respective resins using low-high hydrogen fluoride method (Houghten et al., 1986; Tam et al., 1983). Peptide mixtures were extracted with water or dilute acetic acid and the solutions lyophilized. **Competitive ELISA:** The competitive ELISA method used here is a modification of the direct ELISA technique described previously (Appel et al., 1990), differing only in the mAb addition step. Briefly, microtiter plates were coated with the antigenic peptide (β-endorphin) at a concentration of 100 pmole/50 µl. After blocking, 25 µl of a 2.5 mg/ml solution of each peptide mixture of the SPCL was added, followed by 25 µl of 50 ng/ml mAb 3E7 (25 µl/well). The mAb was added to a fixed dilution previously determined to effectively compete for binding of the antigenic peptide in solution with the antigenic peptide on the plate. The remaining steps are the same as for direct ELISA. The concentration of each peptide mixture necessary to inhibit 50% of the mAb binding to the control peptide on the plate (IC_{50}) was determined by serial dilutions of the peptide mixture. These were found to be consistent in at least three separate determinations. Sigmoidal curves were obtained using Graphpad (ISI Software, San Diego, CA). Intra- and inter-assay variations were 10% and 25%, respectively.

TABLE I

Iterative process of YGXXXX-NH_2 from Fig. 1 for the identification of the antigenic determinant of β-endorphin recognized by mAb 3E7[a]

A		B		C		D	
YGOXXX-NH_2	IC_{50} (nM)	YGGOXX-NH_2	IC_{50} (nM)	YGFOXX-NH_2	IC_{50} (nM)	YGGFOX-NH_2	IC_{50} (nM)	YGGFMO-NH_2	IC_{50} (nM)
YGFXXX-NH_2	400	YGGFXX-NH_2	48	YGFLXX-NH_2	400	YGGFMX-NH_2	6.6	YGGFMT-NH_2	3.2
YGGXXX-NH_2	1000	YGGLXX-NH_2	1160	YGFWXX-NH_2	400	YGGFIX-NH_2	7.2	YGGFMS-NH_2	4.3
YGYXXX-NH_2	3000	YGGWXX-NH_2	6840	YGFMXX-NH_2	1400	YGGFLX-NH_2	8.8	YGGFME-NH_2	4.4
YGHXXX-NH_2	3000	YGGIXX-NH_2	18220	YGFIXX-NH_2	1520	YGGFTX-NH_2	22	YGGFML-NH_2	6.0
YGAXXX-NH_2	14000	YGGMXX-NH_2	26680	YGFTXX-NH_2	1660	YGGFSX-NH_2	28	YGGFMR-NH_2	6.0
YGWXXX-NH_2	34000	YGGYXX-NH_2	98220	YGFFXX-NH_2	2300	YGGFFX-NH_2	28	YGGFMN-NH_2	6.2
YGVXXX-NH_2	46000	YGGRXX-NH_2	179780	YGFSXX-NH_2	3420	YGGFQX-NH_2	28	YGGFMK-NH_2	6.3
YGSXXX-NH_2	94000	YGGGXX-NH_2	253500	YGFCXX-NH_2	3580	YGGFYX-NH_2	34	YGGFMI-NH_2	6.3
YGIXXX-NH_2	178000	YGGTXX-NH_2	399620	YGFRXX-NH_2	3640	YGGFHX-NH_2	34	YGGFMA-NH_2	6.7
YGCXXX-NH_2	201000	YGGCXX-NH_2	400000	YGFYXX-NH_2	3760	YGGFEX-NH_2	36	YGGFMH-NH_2	7.1
YGLXXX-NH_2	256000	YGGVXX-NH_2	517060	YGFEXX-NH_2	3840	YGGFNX-NH_2	38	YGGFMP-NH_2	7.1
YGPXXX-NH_2	288000	YGGQXX-NH_2	732040	YGFDXX-NH_2	3960	YGGFVX-NH_2	50	YGGFMV-NH_2	7.4
YGNXXX-NH_2	463000	YGGAXX-NH_2	800000	YGFQXX-NH_2	4140	YGGFGX-NH_2	56	YGGFMD-NH_2	7.5
YGMXXX-NH_2	470000	YGGPXX-NH_2	1081160	YGFVXX-NH_2	4880	YGGFDX-NH_2	56	YGGFMG-NH_2	7.7
YGTXXX-NH_2	516000	YGGHXX-NH_2	1232200	YGFNXX-NH_2	5060	YGGFAX-NH_2	62	YGGFMQ-NH_2	8.3
YGKXXX-NH_2	532000	YGGSXX-NH_2	1690580	YGFHXX-NH_2	5300	YGGFWX-NH_2	64	YGGFMM-NH_2	11.3
YGDXXX-NH_2	1750000	YGGNXX-NH_2	1750000	YGFAXX-NH_2	5860	YGGFCX-NH_2	72	YGGFMY-NH_2	16.0
YGQXXX-NH_2	1750000	YGGEXX-NH_2	1750000	YGFGXX-NH_2	7160	YGGFKX-NH_2	146	YGGFMF-NH_2	25.8
YGRXXX-NH_2	1750000	YGGKXX-NH_2	1750000	YGFPXX-NH_2	7980	YGGFRX-NH_2	246	YGGFMC-NH_2	ND
YGEXXX-NH_2	1750000	YGGDXX-NH_2	1750000	YGFKXX-NH_2	14400	YGGFPX-NH_2	352	YGGFMW-NH_2	ND

[a]See section b 2. ND, not determined. Notations – NH_2 refer to C-terminal amides.

discrimination in favor of the tightest binding phage. More stringent phage selection conditions (e.g., less mAb) was shown recently to favor the selection of peptides having the highest affinities (Barrett, et al., 1992).

In the second peptide library approach, nearly two million pentapeptides on beads were reacted with mAb 3E7 (Lam et al., 1991), and the beads that bound the mAb identified with a mAb-linked color reagent. Six individual positive beads were separated with microforceps and their attached peptides sequenced. One of the peptides found (YGGFQ-OH; K_i = 15 nM) had a binding affinity similar to the reference control peptide YGGFL-OH (K_i = 18 nM). The expected determinant sequence (YGGFM) and the reference control sequence (YGGFL) were not found. This may be due to the fact that the beads bearing those peptides were not chosen for sequencing, or because those peptides were conformationally unavailable to the mAb as a result of interaction with the resin.

(d) Conclusions

The SPCL approach tests peptide mixtures in free solution, thus circumventing limitations inherent in approaches in which peptides are bound to beads or fused to phage. The SPCL described here consists of 400 different mixtures of hexapeptides, each mixture made up of 130 321 different hexapeptides. At a total concentration of 1.0 mg/ml, the concentration of each of the individual peptides making up these peptide mixtures is approximately 9.8 nM, sufficient to detect activity in assays routinely used in the study of antigen/antibody, receptor/ligand, and enzyme/substrate interactions, etc. In the current study of mAb 3E7, the sequence YGGFMT was found to be a high affinity peptide out of more than 52 million different hexapeptides. This exactly matches the first six aa of the β-endorphin sequence used for the preparation of mAb 3E7 (Gramsch et al., 1983; Dandekar et al., 1985). This peptide library approach, and the others briefly reviewed here, complement existing rational drug design methods currently used to study such peptide/receptor interactions as X-ray crystallography, nuclear magnetic resonance, and computer modeling, since large numbers of optimal peptide sequences can be rapidly identified for a particular receptor system of interest. We believe that the use of SPCLs as described greatly facilitates, and fundamentally changes, the timeframe for drug discovery, the development of immunodiagnostics and, basic research involving peptides.

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Acetalins: Opioid receptor antagonists determined through the use of synthetic peptide combinatorial libraries

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ABSTRACT **A synthetic peptide combinatorial library made up of 52,128,400 hexapeptides, each having an acetyl group at the N terminus and an amide group on the C terminus, was screened to find compounds able to displace tritiated [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin from μ opioid receptor binding sites in crude rat brain homogenates. Individual peptides with μ receptor affinity were found using an iterative process for successively determining the most active peptide mixtures. Upon completion of this iterative process, the three peptides with the highest affinity were Ac-RFMWMT-NH_2, Ac-RFMWMR-NH_2, and Ac-RFMWMK-NH_2. These peptides showed high affinity for μ and κ_3 opioid receptors, somewhat lower affinity for δ receptors, weak affinity for κ_1 receptors, and no affinity for κ_2 receptors. They were found to be potent μ receptor antagonists in the guinea pig ileum assay and relatively weak antagonists in the mouse vas deferens assay. These peptides represent a class of opioid receptor ligands that we have termed acetalins (*acetyl* plus *enkephalin*).**

Development of opioid compounds with high specificity for each opioid receptor type (μ, δ, or κ) and subtype continues to be an important goal in opioid pharmacology. The three receptor types possess analgesic properties; however, the type of pain inhibited and the secondary functions of the receptors have been shown to differ among the three receptor types. The μ receptor has generally been regarded as the receptor type associated with pain relief and has been shown to be potent in regulating thermal pain (1). Nonanalgesic effects mediated by the μ receptor include respiratory depression (for review, see ref. 2), inhibition of intestinal motility (3), antidiuresis (4), suppression of the immune system (5), and (most importantly for therapeutic considerations) physical dependence (6). The δ receptor is also associated with thermal analgesia (1, 7), but with reduced effects on respiration (8) and addiction (9, 10). The κ receptor, in contrast, is most potent in the mediation of analgesia in response to pain induced by chemical stimuli (11, 12). It has also been shown to induce diuresis (13), food intake (14), and sedation (15, 16) and to regulate neuroendocrine synthesis and/or release (for review, see ref. 12). The κ receptor has a much reduced potential for dependence (6) but has been shown to be associated with dysphoric (17) and psychomimetic (18) effects. Such differences in receptor function encourage the search for drugs that produce analgesia without deleterious side effects.

Both receptor-specific opioid agonists and antagonists are useful pharmacological tools and have potential as therapeutic agents. Specific antagonists are required for the determination of effects mediated by specific receptor types and subtypes. In recent years, considerable progress has been made in the development of selective opioid receptor peptide ligands having agonist or antagonist properties. These were determined using various design strategies, including substitution of natural and nonproteinogenic amino acids, conformational restriction, and the bivalent ligand approach (for reviews, see refs. 19 and 20).

The recent development of peptide libraries allows for a more systematic approach for the determination of additional peptide ligands (21–29). Tens to hundreds of millions of peptide sequences can now be rapidly screened to determine peptide sequences that strongly interact with receptors, antibodies, etc. While the various library approaches each have their own specific merits, synthetic peptide combinatorial libraries (SPCLs) (21, 22, 30) differ from other peptide libraries in that the peptide mixtures are not support-bound and thus can be used directly in solution with any assay system. For receptor binding studies, the SPCL approach offers the advantage of not being limited to studies in which pure soluble receptors are available.

Earlier studies in this laboratory have shown that the SPCL approach can be used for the rapid determination of peptides that bind strongly to μ opioid receptors (31, 32). In these initial studies, an SPCL composed of peptides having a free amino group at the N terminus, when used in conjunction with an iterative selection process, enabled the determination of individual peptides that inhibited binding of tritiated [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAMGO) to μ receptors in crude rat brain homogenates. The most effective peptides found were related to the naturally occurring enkephalins and had activities in the range of 20–40 nM. In the present study, an N-terminal acetylated SPCL, used successfully in a variety of earlier studies for the identification of antigenic determinants (21, 22, 30, 33, 34) and for the development of antimicrobial peptides (21, 22, 35), has been employed in the determination of additional peptide ligands found to be potent inhibitors of DAMGO binding at the μ receptor. The peptide library used in this study is made up of 400 mixtures, each composed of 130,321 hexapeptides (19^4). In total, the library contains 52,128,400 hexapeptides (400 × 130,321). The library can be represented by the formula $Ac\text{-}O_1O_2X_3X_4X_5X_6\text{-}NH_2$, in which the first two positions (O_1 and O_2) are individually defined using the 20 natural L-amino acids (i.e., AA, AC, AD, . . . , YV, YW, YY). The remaining four positions (X_3, X_4, X_5, and X_6) consist of equimolar mixtures of 19 of the 20 natural L-amino acids (cysteine omitted). The 400 mixtures making up this SPCL were screened for their ability to inhibit the specific binding of ^{3}H-labeled DAMGO to μ receptors in crude rat brain homogenates. The most active mixtures found from the initial screening were further defined in an iterative selection process, which sequentially defined the four mixture positions

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Abbreviations: SPCL, synthetic peptide combinatorial library; DAMGO, [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin; MVD, mouse vas deferens; GPI, guinea pig ileum; NIDA, National Institute of Drug Abuse.

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(i.e., $O_1O_2O_3XXX\text{-}NH_2$, $O_1O_2O_3O_4XX\text{-}NH_2$, $O_1O_2O_3O_4O_5X\text{-}NH_2$, and $O_1O_2O_3O_4O_5O_6\text{-}NH_2$) (36). The iterative process for one of these mixtures is described here. The μ, δ, κ_1, κ_2, and κ_3 receptor binding affinities of three of the individual peptides thus determined and their antagonist potencies in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays are presented.

MATERIALS AND METHODS

Preparation of SPCL. Assembly of the SPCL and preparation of mixtures for the iterative process have been described in detail (21, 22). XXXX-resin was prepared using a process of division, coupling, and recombination of individual resins. The XXXX-resin was then divided into 400 equal portions (each containing 250 mg). Two individual amino acids, O_1 and O_2, were added using simultaneous multiple peptide synthesis (37), an adaptation of Merrifield's solid-phase method (38). After acetylation, deprotection, and cleavage from the resins, each of the 400 peptide mixtures was extracted with water to yield a final peptide concentration of 1–3 mg/ml.

HPLC and Purification. Analytical HPLC was carried out using a Beckman–Altex model 421 HPLC system and dual model 110a pumps with a Vydac (Hesperia, CA) C_{18} column (25 cm × 4.6 mm) and Hitachi 100-20 spectrophotometer. Chromatograms were recorded and peak heights were integrated on a Shimadzu CR3A Chromatopac integrator. Individual peptides were purified using a Waters Milliprep 300 preparative HPLC modified with a Gilson model 232 preparative autosampler and Foxy fraction collector. Pure fractions (determined using analytical HPLC) were pooled and lyophilized.

Opioid Receptor Binding Assay. Preparation of rat brain membranes and the receptor binding assay were carried out as described (32). Each tube in the screening assay contained 0.5 ml of membrane suspension, 7 nM ^{3}H-labeled DAMGO [specific activity, 36 Ci/mmol (1 Ci = 37 GBq), obtained from National Institute of Drug Abuse (NIDA) repository, prepared by Multiple Peptide Systems (San Diego)], a peptide mixture (0.08 mg/ml), and 50 mM Tris·HCl (pH 7.4) in a total volume of 0.65 ml. Competition curves were generated using serial dilutions of the peptide mixtures. IC_{50} values were determined for active mixtures using the software GRAPHPAD (ISI, San Diego).

GPI and MVD Bioassays. The GPI (39) and MVD (40) bioassays were carried out as reported in detail elsewhere (41, 42). A logarithmic dose–response curve was obtained with [Leu^5]enkephalin for each ileum and vas preparation, and the IC_{50} value was determined. K_e values for antagonists were determined from the ratio of IC_{50} values obtained with [Leu^5]enkephalin in the presence and absence of a fixed antagonist concentration (43).

RESULTS

The 400 peptide mixtures in the SPCL ($Ac\text{-}O_1O_2XXXX\text{-}NH_2$) were assayed to determine their ability to inhibit the binding of ^{3}H-labeled DAMGO to crude rat brain homogenates (Fig. 1). Eighty percent of the mixtures inhibited <50% of DAMGO binding at the concentration screened (0.08 mg/ml). IC_{50} values for mixtures that showed the greatest inhibition in the initial screening were determined. The most effective inhibitors of tritiated DAMGO binding were found to be mixtures containing arginine at the first (N-terminal) position (Table 1). Ac-RWXXXX-NH_2, with an IC_{50} value of 2128 nM, and Ac-RFXXXX-NH_2, with an IC_{50} value of 2347 nM, were found to have the greatest ability to inhibit ^{3}H-labeled DAMGO binding (the difference between these two IC_{50} values was not significant). Ac-FRXXXX-NH_2 (IC_{50} = 3034 nM) and Ac-WRXXXX-NH_2 (IC_{50} = 6153 nM) were the most effective mixtures with amino acids other than arginine at the

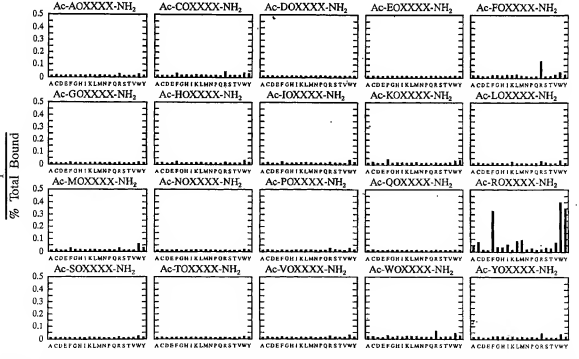

FIG. 1. Initial screening of the SPCL ($Ac\text{-}OOXXXX\text{-}NH_2$) for the ability to inhibit the binding of [^{3}H]DAMGO to crude rat brain homogenates. Individual bar graphs are segregated by the first amino acid (O_1), with individual bars in each graph representing the 20 amino acids making up the second position (O_2). The *y* axis represents the reciprocal of the percentage of [^{3}H]DAMGO bound.

Table 1. Inhibition of [^{3}H]DAMGO by peptide mixtures derived from the acetylated SPCL

Ac-ROXXXX-NH$_2$	IC$_{50}$, nM	Ac-RFOXXX-NH$_2$	IC$_{50}$, nM	Ac-RFMOXX-NH$_2$	IC$_{50}$, nM	Ac-RFMWOX-NH$_2$	IC$_{50}$, nM	Ac-RFMWMO-NH$_2$	IC$_{50}$, nM
Ac-RWXXXX-NH$_2$	2128 ±513	Ac-RFMXXX-NH$_2$	723 ±252	Ac-RFMWXX-NH$_2$	174 ±18	Ac-RFMWMX-NH$_2$	30 ±5	Ac-RFMWMK-NH$_2$	5 ±3
Ac-RFXXXX-NH$_2$	2347 ±571	Ac-RFLXXX-NH$_2$	1252 ±490	Ac-RFMSXX-NH$_2$	541 ±68	Ac-RFMWVX-NH$_2$	54 ±6	Ac-RFMWMT-NH$_2$	5 ±1
Ac-RTXXXX-NH$_2$	7906 ±2236	Ac-RFIXXX-NH$_2$	1432 ±398	Ac-RFMAXX-NH$_2$	595 ±95	Ac-RFMWQX-NH$_2$	83 ±23	Ac-RFMWMR-NH$_2$	6 ±1
Ac-RYXXXX-NH$_2$	8164 ±2673	Ac-RFXXXX-NH$_2$	2701 ±707	Ac-RFMGXX-NH$_2$	641 ±73	Ac-RFMWXX-NH$_2$	106 ±7	Ac-RFMWMS-NH$_2$	7 ±3
Ac-RVXXXX-NH$_2$	8380 ±1056	Ac-RFWXXX-NH$_2$	3009 ±278	Ac-RFMIXX-NH$_2$	730 ±87	Ac-RFMWIX-NH$_2$	108 ±7	Ac-RFMWMG-NH$_2$	10 ±1
Ac-RMXXXX-NH$_2$	11060 ±2189	Ac-RFFXXX-NH$_2$	3031 ±704	Ac-RFMVXX-NH$_2$	790 ±54	Ac-RFMWYX-NH$_2$	125 ±4	Ac-RFMWMN-NH$_2$	11 ±3
Ac-RAXXXX-NH$_2$	13836 ±969	Ac-RFGXXX-NH$_2$	4484 ±1255	Ac-RFMMXX-NH$_2$	870 ±128	Ac-RFMWLX-NH$_2$	180 ±23	Ac-RFMWMQ-NH$_2$	12 ±3
Ac-RGXXXX-NH$_2$	14555 ±3534	Ac-RFYXXX-NH$_2$	7204 ±1369	Ac-RFMRXX-NH$_2$	882 ±107	Ac-RFMWRX-NH$_2$	187 ±7	Ac-RFMWMA-NH$_2$	15 ±4
Ac-RCXXXX-NH$_2$	16823 ±2794	Ac-RFHXXX-NH$_2$	7296 ±955	Ac-RFMXXX-NH$_2$	907 ±100	Ac-RFMWAX-NH$_2$	204 ±5	Ac-RFMWMV-NH$_2$	17 ±7
Ac-RIXXXX-NH$_2$	19629 ±6022	Ac-RFNXXX-NH$_2$	7377 ±4658	Ac-RFMCXX-NH$_2$	915 ±12	Ac-RFMWKX-NH$_2$	210 ±15	Ac-RFMWMY-NH$_2$	17 ±4
Ac-RHXXXX-NH$_2$	24134 ±6388	Ac-RFRXXX-NH$_2$	7980 ±432	Ac-RFMHXX-NH$_2$	927 ±45	Ac-RFMWGX-NH$_2$	313 ±42	Ac-RFMWMM-NH$_2$	18 ±4
Ac-RLXXXX-NH$_2$	28578 ±7222	Ac-RFCXXX-NH$_2$	8792 ±1942	Ac-RFMTXX-NH$_2$	1048 ±52	Ac-RFMWTX-NH$_2$	321 ±26	Ac-RFMWML-NH$_2$	20 ±3
Ac-RPXXXX-NH$_2$	31391 ±6888	Ac-RFQXXX-NH$_2$	11519 ±1691	Ac-RFMNXX-NH$_2$	1169 ±110	Ac-RFMWSX-NH$_2$	327 ±53	Ac-RFMWMH-NH$_2$	20 ±5
Ac-RSXXXX-NH$_2$	38263 ±22941	Ac-RFTXXX-NH$_2$	11561 ±794	Ac-RFMKXX-NH$_2$	1310 ±155	Ac-RFMWWX-NH$_2$	327 ±21	Ac-RFMWMP-NH$_2$	24 ±7
Ac-RQXXXX-NH$_2$	109327 ±25720	Ac-RFAXXX-NH$_2$	12972 ±1624	Ac-RFMQXX-NH$_2$	1375 ±188	Ac-RFMWHX-NH$_2$	348 ±17	Ac-RFMWMX-NH$_2$	24 ±6
Ac-RRXXXX-NH$_2$	116347 ±29932	Ac-RFKXXX-NH$_2$	14003 ±147	Ac-RFMYXX-NH$_2$	1468 ±146	Ac-RFMWFX-NH$_2$	378 ±177	Ac-RFMWMW-NH$_2$	46 ±13
Ac-RNXXXX-NH$_2$	127342 ±36372	Ac-RFSXXX-NH$_2$	14145 ±1642	Ac-RFMDXX-NH$_2$	1908 ±13	Ac-RFMWNX-NH$_2$	454 ±4	Ac-RFMWMI-NH$_2$	67 ±27
Ac-REXXXX-NH$_2$	133230 ±33324	Ac-RFPXXX-NH$_2$	23895 ±6044	Ac-RFMFXX-NH$_2$	2004 ±136	Ac-RFMWPX-NH$_2$	616 ±66	Ac-RFMWMF-NH$_2$	68 ±20
Ac-RDXXXX-NH$_2$	146864 ±84802	Ac-RFVXXX-NH$_2$	30282 ±19881	Ac-RFMLXX-NH$_2$	2166 ±220	Ac-RFMWEX-NH$_2$	795 ±9	Ac-RFMWMC-NH$_2$	79 ±14
Ac-RKXXXX-NH$_2$	194501 ±61212	Ac-RFDXXX-NH$_2$	44588 ±7648	Ac-RFMPXX-NH$_2$	2626 ±204	Ac-RFMWDX-NH$_2$	952 ±143	Ac-RFMWME-NH$_2$	96 ±9
		Ac-RFEXXX-NH$_2$	77533 ±4311	Ac-RFMEXX-NH$_2$	3912 ±44	Ac-RFMWCX-NH$_2$	3185 ±369	Ac-RFMWMD-NH$_2$	109 ±16

IC$_{50}$ values for peptide mixtures, Ac-ROXXXX-NH$_2$, from the SPCL and for peptides defining the third, fourth, fifth, and sixth positions in the iterative process as indicated. The IC$_{50}$ value of the peptide mixture found most effective in the previous mixture is boxed for comparison.

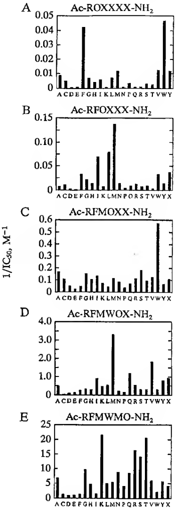

FIG. 2. Reciprocal of IC$_{50}$ values obtained for peptide mixtures, Ac-ROXXXX-NH$_2$, from the SPCL (*A*), and for peptide mixtures defining the third (*B*), fourth (*C*), fifth (*D*), and sixth (*E*) positions in the iterative process. Individual bars are labeled on the *x* axis by the 20 amino acids (single-letter code) used to define the particular position (O). The most effective peptide mixture from the previous iterative step is labeled X.

first position. The iterative selection process carried out for the mixture Ac-RFXXXX-NH$_2$ is reported here. Iterations for other initially less active cases will be reported elsewhere.

Twenty peptide mixtures, each made up of 6859 hexapeptides (19^3), were synthesized to define the third position of Ac-RFXXXX-NH$_2$. These mixtures are represented by Ac-RFOXXX-NH$_2$ (i.e., Ac-RFAXXX-NH$_2$ through Ac-RFYXXX-NH$_2$). Three mixtures had binding affinities greater than that of Ac-RFXXXX-NH$_2$: Ac-RFMXXX-NH$_2$ (IC$_{50}$ = 723 nM), Ac-RFLXXX-NH$_2$ (IC$_{50}$ = 1252 nM), and Ac-RFIXXX-NH$_2$ (IC$_{50}$ = 1432 nM). The remaining positions of Ac-RFMXXX-NH$_2$ (i.e., X_4, X_5, and X_6) were then defined in sequential order in a similar manner. Binding affinities (IC$_{50}$, mean ± SEM) of the peptide mixtures found for each step of the screening and selection process are shown in Table 1; improvements in binding inhibition made at each iterative step are illustrated in Fig. 2.

Of the 20 mixtures making up Ac-RFMOXX-NH$_2$ (each composed of 361 peptides), 8 exhibited binding affinities

Table 2. Affinity of acetalins for three opioid receptors

Peptide	Receptor	IC_{50}, nM	K_i, nM	STD ratio, K_iSTD/K_i	Hill slope
Ac-RFMWMT-NH_2	μ	1.7	0.8	1.38	1.0
Ac-RFMWMK-NH_2	μ	1.9	0.4	2.75	1.0
Ac-RFMWMR-NH_2	μ	1.1	0.5	2.20	1.2
Ac-RFMWMT-NH_2	δ	3.4	0.9	0.333	1.2
Ac-RFMWMK-NH_2	δ	24.7	5.6	0.054	0.8
Ac-RFMWMR-NH_2	δ	32.4	7.4	0.041	1.1
Ac-RFMWMT-NH_2	κ_1	2411	1108	0.006	0.9
Ac-RFMWMK-NH_2	κ_1	853	392	0.002	0.8
Ac-RFMWMR-NH_2	κ_1	494	227	0.003	0.7
Ac-RFMWMT-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMK-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMR-NH_2	κ_2	>10,000	<0.0001		
Ac-RFMWMT-NH_2	κ_3	1.0	0.6	0.5	1.0
Ac-RFMWMK-NH_2	κ_3	0.7	0.4	0.8	0.9
Ac-RFMWMR-NH_2	κ_3	2.6	1.4	0.21	1.1

Guinea pig brain suspension [1.8 ml, 6.7 mg (wet weight) tissue per ml in Tris·HCl (pH 7.7)] was incubated for 1 hr at 25°C with 100 μl of radioligand and 100 μl of peptide (10^{-5}–10^{-11} M). Membranes were labeled (≈1 nM) with [^{3}H]DAMGO, cyclic [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDE) (where Pen is penicillamine), [^{3}H]U 69,593, [^{3}H]bremazocine in the presence of 100 nM DAMGO, [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET), and U 69,593, and [^{3}H]naloxone benzoylhydrazone in the presence of 100 nM U 69,593 for μ, δ, κ_1, κ_2, and κ_3 receptors, respectively. Nonspecific binding was determined using 1 μM DAMGO, Cl-DPDPE, or U 69,593 for μ, δ, and κ_1 receptors, respectively, or 10 μM bremazocine and naloxone benzoylhydrazone (κ_2 and κ_3 receptors). This data was provided by NIDA (Contract 271-89-8159). STD, standard.

greater than that of Ac-RFMXXX-NH_2. The peptide mixture with the highest affinity in this series (Ac-RFMWXX-NH_2; IC_{50} = 174 nM) was found to displace ^{3}H-labeled DAMGO three times more readily than the next most potent mixture, Ac-RFMSXX-NH_2 (IC_{50} = 541 nM) and five to six times more readily than Ac-RFMXXX-NH_2. Upon defining the fifth position, Ac-RFMWXX-NH_2 (19 peptides in each mixture), three mixtures exhibited inhibiting capabilities greater than that of Ac-RFMWXX-NH_2: Ac-RFMWMX-NH_2 (IC_{50} = 30 nM), Ac-RFMWVX-NH_2 (IC_{50} = 54 nM), and Ac-RFMWQX-NH_2 (IC_{50} = 83 nM). For the final iteration, in which all six positions were defined (Ac-RFMWMO-NH_2), 14 peptides had inhibiting capabilities greater than that of Ac-RFMWMX-NH_2. The four most potent peptides had IC_{50} values below 10 nM: Ac-RFMWMK-NH_2 (IC_{50} = 5 nM), Ac-RFMWMT-NH_2 (IC_{50} = 5 nM), Ac-RFMWMR-NH_2 (IC_{50} = 6 nM), and Ac-RFMWMS-NH_2 (IC_{50} = 7 nM). The IC_{50} value determined for unlabeled DAMGO was 7 nM.

The importance of the N-terminal acetyl and C-terminal amide groups was investigated. Affinities for three peptides synthesized without an acetyl moiety on the N terminus were RFMWMT-NH_2 (IC_{50} = 1799 ± 178 nM), RFMWMK-NH_2 (IC_{50} = 1041 ± 71 nM), and RFMWMR-NH_2 (IC_{50} = 630 ± 92 nM). Affinities of three peptides synthesized with a carboxyl group at the C terminus and an acetyl group at the N terminus were Ac-RFMWMT-COOH (IC_{50} = 352 ± 73 nM), Ac-RFMWMK-COOH (IC_{50} = 164 ± 25 nM), and Ac-RFMWMR-COOH (IC_{50} = 276 ± 51 nM).

The affinities of Ac-RFMWMR-NH_2, Ac-RFMWMK-NH_2, and Ac-RFMWMT-NH_2 for μ, δ, κ_1, κ_2, and κ_3 receptors were determined in specific binding assays (Table 2).

Table 3. Opioid antagonist potencies (K_e values) of peptides in the GPI and MVD assays

Peptide	K_e, nM	
	GPI	MVD
Ac-RFMWMT-NH_2	24.8 ± 6.5	>1000
Ac-RFMWMK-NH_2	2.53 ± 0.41	955 ± 201
Ac-RFMWMR-NH_2	2.92 ± 0.62	326 ± 31

Values were determined against [Leu^5]enkephalin as agonist.

These three peptides showed no affinity for κ_2 receptors at the highest concentration tested (10 μM) and weak affinity for κ_1 receptors with IC_{50} values of 494 nM, 853 nM, and 2410 nM, respectively. These three peptides, however, displayed high affinities for the κ_3 receptor, with respective IC_{50} values of 2.6 nM, 0.7 nM, and 1.0 nM. Affinities of the three peptides were also high for the μ receptor with IC_{50} values of 1.1 nM, 0.9 nM, and 1.7 nM, respectively. The peptides had somewhat lower affinities for the δ receptor with IC_{50} values of 32.4 nM, 24.7 nM, and 3.4 nM, respectively. Ac-RFMWMR-NH_2 was the only peptide that displayed even modest selectivity for μ receptors [$K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:3:15]. Ac-RFMWMK-NH_2 had a $K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:1:14, and Ac-RFMWMT-NH_2 had close to equal affinities for the μ, κ_3 and δ receptors [$K_i(\mu)/K_i(\kappa_3)/K_i(\delta)$ ratio of 1:1:1]. Differences in IC_{50} values found in this laboratory (Table 1) and those obtained at NIDA (Table 2) may be due to species differences, rat tissue vs. guinea pig tissue, or to the lower concentration of labeled DAMGO used in the NIDA study.

In the μ-receptor-representative GPI assay, Ac-RFMWMR-NH_2 and Ac-RFMWMT-NH_2 were found to be potent μ antagonists of [Leu^5]enkephalin, with K_e values of 2.53 nM and 2.92 nM, respectively. They were, however, >100 times less potent as δ antagonists against [Leu^5]enkephalin in the MVD assay (K_e = 955 nM and 326 nM, respectively) (Table 3). In comparison with the latter two peptides, Ac-RFMWMK-NH_2 had an ≈10 times lower μ antagonist potency in the GPI assay and showed no δ antagonist activity at concentrations up to 1 μM in the MVD assay. The weak δ antagonist potencies of these compounds are in agreement with the fact that they have relatively lower affinities for δ receptors than for μ receptors. Qualitative and quantitative differences observed between the receptor binding data and the bioassay results may reflect the existence of different μ and δ receptor subtype populations in rat brain and in the isolated tissue preparations.

DISCUSSION

In the present study, an SPCL composed of >52 million hexapeptides, when linked with an iterative selection and

enhancement process, enabled the discovery of a class of acetylated peptide sequences capable of displacing ^{3}H-labeled DAMGO from its receptor binding sites. The peptides found in the current study are not listed in the Registry produced by Chemical Abstracts Service obtained through The Scientific and Technical Information Network as known peptides or as part of known protein sequences. We have termed these peptides "acetalins" due to their opioid binding characteristics and the presence of the N-terminal acetyl group (acetalins = *acet*ylated enkep*halins*).

The presence of the acetyl group on the N terminus is critical for the binding of these peptides, since analogues lacking the acetyl moiety exhibited very weak binding (IC_{50} in the micromolar range). An amide group on the C terminus is also preferred for binding, with a 10- to 30-fold decrease in affinity when the amide group was replaced by a carboxyl group (IC_{50} = 100–300 nM).

In each of the iterative steps, defining a position with an aspartic acid or glutamic acid residue resulted in very weak activity. Mixtures containing either of these residues were always among the least active of the 20 peptides in an iteration. This observation is in agreement with reports that the presence of an aspartic acid or glutamic acid residue in deltorphin infers high selectivity for the δ receptor, apparently by inhibiting binding to the μ receptor (44, 45).

The three peptides Ac-RFMWMT-NH_2, Ac-RFMWMK-NH_2, and Ac-RFMWMR-NH_2 are opioid receptor ligands with high affinity for μ and κ_3 receptors. They show negligible preference for μ over κ_3 receptors. It has been suggested, however, that the κ_3 receptor subtype can be considered an isoform of the μ receptor (12). The concentration of tritiated DAMGO used in the assay is expected to occupy the κ_3 receptors (12). This concentration potentially reduces the ability to discriminate between μ and κ_3 sites. The concentration of label used, however, was constrained by the limitations of the filtration system employed (22). These compounds, therefore, appear not to be highly selective for a single opioid receptor type or subtype. It is interesting to note that the acetalins have high affinity for μ and κ_3 receptors, even though their N-terminal group is acetylated. A positively charged N-terminal amino group is considered to be critical for binding to opioid receptors and is generally thought to be involved in an electrostatic interaction with a negatively charged receptor moiety. It is possible that the positively charged side chain of the Arg^1 residue in these peptides plays a role similar to the N-terminal α-amino functionality in classical opioid peptides in opioid receptor binding.

In a separate study, an N-acetylated C-terminal amidated SPCL compound entirely of D-amino acids was examined. We were able to identify potent inhibitors of DAMGO composed solely of D-amino acids. The peptides derived from this all D-amino acid library had sequences that contained similarities to, but differed from, those found in the present study. The most potent of the sequences identified was Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Lys (IC_{50} = 16 nM; unpublished data). The current report of the potent N-acetylated peptide sequences describes the use of peptide libraries to determine additional sequences that bind to a membrane receptor. The acetalins, though potent inhibitors of binding at the μ, δ, and κ_3 receptor sites, must be considered lead compounds.

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